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Research Paper

Fuzhuan brick tea extract ameliorates obesity-induced skeletal muscle atrophy by alleviating mitochondrial dysfunction in mice

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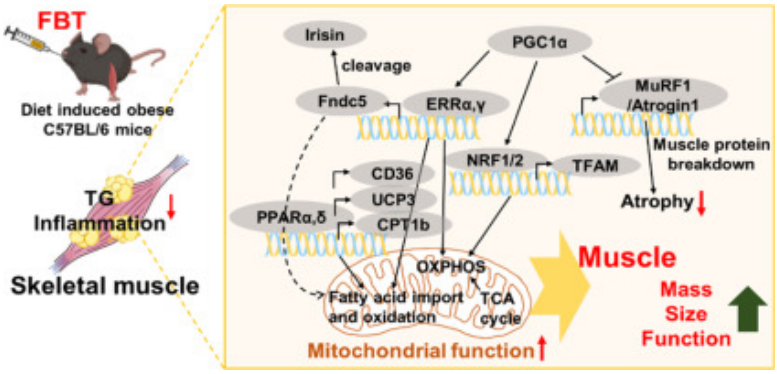
Abstract

Fuzhuan brick tea (FBT) is a post-fermented tea fermented by the fungus *Eurotium cristatum* and is mainly produced in Hunan Province, China. Our previous study revealed that FBT extract prevents obesity by increasing energy expenditure and mitochondrial content in mice. Therefore, in this study, we hypothesized that FBT extract could be effective in alleviating obesity-induced muscle atrophy by addressing mitochondrial dysfunction, and aimed to explore the underlying molecular mechanism of FBT extract in high-fat diet-induced obese mice. FBT extract increased skeletal muscle weight and size, myosin heavy chain isoforms, and muscle performance in obese mice. Additionally, FBT extract reduced obesity-induced intramuscular lipids, skeletal muscle inflammation, and the expression of skeletal muscle atrophy markers, and increased the expression of fibronectin type III domain-containing protein 5 in skeletal muscles. Obesity-induced skeletal muscle mitochondrial dysfunction was improved by FBT extract as analyzed through mitochondrial morphology, fatty acid oxidation, respiratory chain complexes, and mitochondrial dynamics and biogenesis. Epigallocatechin, a major bioactive compound in FBT extract, attenuated palmitic acid-induced muscle atrophy by regulating

mitochondrial functions in C2C12 cells. In conclusion, FBT extract may prevent obesity-induced muscle atrophy by alleviating mitochondrial dysfunction in mice.

Graphical abstract



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Keywords

Fuzhuan brick tea; obesity-induced muscle atrophy; muscle inflammation; mitochondrial dysfunction; epigallocatechin

1. INTRODUCTION

Fuzhuan brick tea (FBT), originating from Hunan Province, China, is a post-fermented tea that undergoes fermentation by the fungus *Eurotium cristatum*. The process of fungal fermentation introduces intricate biochemical transformations in compounds found in the tea, including catechins, amino acids, proteins, organic acids, fatty acid amides, lipids, and polysaccharides. This gives rise to the distinctive aroma, flavor, and potential health advantages associated with FBT [1]. FBT extract exhibits various pharmacological benefits against metabolic diseases, including obesity [2], hyperlipidemia [3], and hyperglycemia [4], and possesses antibacterial [5], anti-colitis [6], and anti-photoaging [7] activities along with regulation of gastrointestinal functions [8].

Obesity is characterized by abnormal or excessive accumulation of fat in the body and is accompanied by various health risks. Muscle composition changes due to obesity, which may lead to 'obesity-induced muscle atrophy', indicated by excess weight and reduced muscle mass or strength simultaneously [9]. The pathogenesis of obesity-induced muscle atrophy involves several mechanisms. Obesity results in low-grade chronic skeletal muscle inflammation, which in turn interferes with anabolic growth of skeletal muscles, leading to metabolic reprogramming of skeletal muscles [10,11]. In addition, accumulation of intramuscular lipids in obesity impairs the functions of

mitochondria and skeletal muscles, which not only impacts energy production but also affects mitochondrial dynamics, involving biogenesis, fusion, and fission processes [12,13]. Mitochondrial dysfunction contributes to the accumulation of reactive oxygen species, alters the functions of myofibrils and motor neurons, and impairs muscle regeneration [14]. A previous study revealed that obese mice exhibited heightened mitochondrial fission in their skeletal muscles, leading to perturbed mitochondrial dynamics. Mitochondrial dysfunction in skeletal muscles led to reduced mitochondrial biogenesis and mitochondrial DNA content and the consequent fatty acid oxidation disorder [15].

Recently, the beneficial effects of FBT extract on the regulation of lipid metabolism have gained worldwide acceptance. Studies have demonstrated anti-obesity effects of FBT extract in both humans and experimental models, such as 3T3-L1 adipocytes [2], *Caenorhabditis elegans* [1], and mice [16]. Our previous study revealed that FBT extract prevents obesity by increasing the mitochondrial content, resulting in thermoregulation in adipocytes [17]. Therefore, in this study, we hypothesized that FBT extract could be effective in alleviating obesity-induced muscle atrophy by addressing mitochondrial dysfunction, and aimed to explore the underlying molecular mechanism of FBT extract in high-fat diet (HFD)-induced obese mice.

2. MATERIALS AND METHODS

2.1. Sample preparation

FBT extract was sourced from Hunan Tea Group Co., Ltd. (Hunan, China). In summary, the FBT underwent a series of processing steps. Initially, it was subjected to triple extraction with 75°C hot water for 1 hour each time. The resulting extract was subsequently concentrated under vacuum after filtration and then subjected to freeze-drying. Ultimately, the desiccated FBT extract was preserved at -20°C until its intended utilization. Epigallocatechin (EGC) was procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

The execution of all animal experiments adhered to the directives set forth by the Institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI-IACUC, KFRI-M−19013). Four-week-old male C57BL/6 mice were housed in a controlled environment featuring a 12-hour light/12-hour dark cycle, consistent temperature (21–25°C), and humidity (50%–60%), with ad libitum access to food and water. Following a one-week acclimation period, the mice were categorized into four groups (n = 10/group): chow diet along with oral administration of vehicle (0.2 mL of distilled water) as a control, HFD (Research Diets, Inc., New Brunswick, NJ, USA; 60% calories as fat) combined with oral administration of vehicle, and HFD coupled with oral administration of FBT extract (100 mg/kg or 200 mg/kg body weight) for 12 weeks. At the end of the experimental period, all mice were fasted for 6 hours, anesthetized with 2% isoflurane, and sacrificed. Following this, blood samples were promptly collected, and measurements were taken after the removal of skeletal muscle tissues. Lipids from the gastrocnemius (gastroc) muscle were extracted using the method described by Folch et al.

[18]. For the assessment of triglyceride (TG) levels in the gastroc muscle, commercially available kits from Abcam (Cambridge, MA, USA) were employed. Serum irisin levels were analyzed using a commercial kit from Biovendor R&D (Brno, Czech Republic).

2.3. Treadmill and grip strength test

Treadmill and grip strength tests were conducted in the last week of the experiment. All mice were adapted to running on a treadmill (Daejong Instrument Industry, Seoul, Korea) for 2 days, and then running distance and time were measured. Detailed experimental conditions were implemented with reference to the previous study [19]. Grip strength was assessed five times using a grip strength test machine (model GS3, Bioseb, Vitrolles, France) in accordance with the manufacturer's instructions, and the outcome was normalized by body weight. The average value was computed, while the maximum and minimum values were excluded from consideration.

2.4. Hematoxylin and eosin (H&E) staining

Fresh gastroc muscle was fixed in 4% formaldehyde and embedded in paraffin, from which 4μm sections were prepared. These sections underwent deparaffinization, rehydration, and staining with H&E-phloxine solution. Subsequently, images were acquired using an Olympus BX51 microscope, and cross-sectional area quantification was performed using IMT iSolution DT 9.2 software.

2.5. Transmission electron microscopy (TEM)

Gastroc muscle was initially fixed in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. Following this fixation, the muscle specimens were subsequently post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, 19152) for 1 h. The tissues were embedded in pure Epon 812 mixture after dehydration in a series of ethanol:water mixtures and infiltration in a propylene oxide: epon mixture series. Using a model MT-X ultramicrotome (RMC, Tucson, AZ, USA), ultra-thin sections (~70 nm) were meticulously sliced and then collected on 100 mesh copper grids. Following the staining procedure with 2% uranyl acetate and 0.2% lead citrate, Cryo-TEM (JEM-1400 Plus, 120 kV) and Bio-HVEM (JEM1000BEF, 1000 kV) (Jeol, Japan) were employed for imaging purposes.

2.6. Mitochondria analysis

Mitochondria were isolated using a Mitochondria Isolation Kit (Thermo Scientific, Rockford, IL, USA), and the activity of Mitochondrial Complex I was assessed in the gastroc muscle tissues using a Complex I Enzyme Activity Microplate Assay Kit (Abcam). Citrate synthase activity and ATP content were determined in homogenized gastroc muscle tissues extracted with RIPA buffer, using a Citrate Synthase Assay Kit (Sigma-Aldrich) and an ATP luminescence detection assay system (ATPlite, PerkinElmer, Waltham, MA, USA), respectively.

2.7. Cell culture

C2C12 myoblast cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO2 atmosphere, and for differentiation, they were cultured in DMEM with 2% horse serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Samples of FBT extract and EGC were treated for 24 h with 0.5 mM palmitic acid (PA) on either day 3 or day 4 of the differentiation process. PA and bovine serum albumin (BSA) stock solutions were prepared as previously described [20].

2.8. Oxygen consumption rate (OCR) measurement

Measurement of mitochondrial OCR in cultured muscle cells was conducted using the Seahorse Bioscience XF96 platform (Seahorse Bioscience, North Billerica, MA, USA).

Briefly, C2C12 cells were seeded in XF96 cell culture microplates and treated with a differentiation medium for 3 days. Thereafter, treatment was performed with or without PA, FBT extract, and EGC for 24 h. Subsequently, after replacing the cell culture medium with the assay medium, the experiment was conducted based on previous study [21].

2.9. Immunofluorescence and quantification of myotubes

Myotubes were subjected to fixation using 4% formaldehyde for 30 min, subsequent permeabilization with 0.05% saponin, blocking with 1% BSA in PBS, and finally, overnight staining with a total myosin heavy chain (MHC) antibody (Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA, USA). at 4°C. Subsequent to the PBS rinsing, the myotubes were subjected to a 30-min incubation at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG (dilution 1:500, Cell Signaling Technology, Beverly, MA, USA), followed by a 1-min incubation at room temperature with 4′,6-diamidino-2-phenylindole (DAPI, dilution 1:10,000). Images were acquired and analyzed using the Olympus IX71 microscope and Olympus DP controller 3.1.1 software, respectively.

2.10. Western blot analysis

The protein extraction from skeletal muscle tissues was conducted using RIPA assay buffer, and the protein concentration of the resulting supernatant was quantified utilizing a Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA). The total protein was separated using SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was then blocked with a solution of 5% skim milk and 0.1% Tween 20 in Tris-buffered saline for 1 h at room temperature. Following an overnight incubation at 4°C with primary antibodies, the membranes were subjected to washing and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Immunodetection was performed using an enhanced chemiluminescence detection reagent (Bio-Rad). Information on primary antibodies is as follows: Antibodies against total MHC, MHC-I, MHC-IIa, and MHC-IIb were purchased from the DSHB. Anti-Vinculin antibody was obtained from Sigma-Aldrich. Antibodies against tumor necrosis factor-α (TNFα), F-box protein (MAFbx/Atrogin1), muscle RING-finger protein 1 (MuRF1), fibronectin type III domain-containing 5 (FNDC5), oxidative

phosphorylation (OXPHOS), peroxisome proliferator-activated receptor (PPAR)γ coactivator 1α (PGC1α), mitochondrial transcription factor A (TFAM) were obtained from Abcam. Antibodies against phospho-Jun N-terminal kinase (JNK), JNK, phospho-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, phospho-c-Jun, c-Jun, phospho-nuclear factor kappa B (NFκB), NFκB, phospho-signal transducer and activator of transcription 3 (STAT3), STAT3, phospho-AMP-activated protein kinase (AMPK), AMPK, and nuclear factor erythroid 2-related factor 2 (NRF2) were obtained from Cell Signaling Technology. Anti-interleukin-6 (IL-6), mitofusin 1 and 2 (Mfn1 and Mfn2), optic atrophy type 1 (OPA1), dynamin-related protein (Drp1), fission protein 1 (Fis1) estrogen-related receptor (ERR)α, ERRγ, nuclear respiratory factor 1 (NRF1) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All dilutions were prepared at 1:1000, except for the MHC-IIb antibody, which was diluted at 1:200.

2.11. Quantitative real-time polymerase chain reaction (PCR)

Gastroc muscle tissues were processed for total RNA extraction using the Qiagen RNeasy Fibrous Tissue Mini Kit (QIAGEN Inc., Hilden, Germany). Subsequently, cDNA was synthesized from the total RNA using the ReverTra Ace® quantitative reverse transcription-polymerase chain reaction (qPCR RT) master kit (Toyobo Co., Ltd., Osaka, Japan). Quantitative PCR was carried out utilizing SYBR Green real-time PCR Master Mix (Toyobo Co., Ltd.) along with specific forward/reverse primers, employing the ViiA7 PCR system (Applied Biosystems, Foster City, CA, USA). The cDNA was utilized as a template in a 20 μL reaction mixture, which underwent an initial step at 95°C for 5 min, followed by 40 amplification cycles (95°C for 5 s, 55°C for 10 s, and 72°C for 15 s). The mRNA levels were then normalized to 18S.

2.12. Statistical analysis

The data are presented as the mean ± SEM or SD, and statistical analyses were conducted using GraphPad Prism software, version 8.3.1 (San Diego, CA, USA). Statistical comparisons between the two groups were performed using an unpaired *t*-test, while comparisons involving more than two groups were assessed using one-way ANOVA followed by Dunnett's test for multiple comparisons.

3. RESULTS

3.1. FBT extract increased the weight and size of skeletal muscles and MHC isoforms in obese mice

To investigate the effect of FBT extract on obesity-induced muscle atrophy, we first measured the weight of body and skeletal muscles of mice. In addition, the cross-sectional area (CSA) of skeletal muscle fibers was measured. The experiment began with 5-week-old mice, with each group having an average body weight of 21 g. Starting from the 3rd week, the HFD-fed mice began gaining significantly more weight compared to the chow-fed mice. However, starting from week 7, the FBT extract groups began to lose weight significantly more than the HFD group. By week 11, there was no

significant difference in weight loss between the 100 FBT and 200 FBT groups (Fig. 1A). Additionally, muscle weight was lower in the HFD group than that in the Chow group, and FBT extract treatment increased skeletal muscle weight in HFD-fed mice (Fig. 1B). We performed most of the analyses on the gastroc muscle, which is a mixed glycolytic-oxidative muscle. The CSA of skeletal muscle fibers of the HFD group was lower than that of the Chow group. However, FBT extract increased the size of muscle fibers of HFD-fed mice (Fig. 1C). We measured the mRNA and protein levels of MHC isoforms in skeletal muscles. The mRNA and protein expression levels of total MHC, MHC-I, MHC-IIa, and MHC-IIb were decreased in HFD-fed mice; however, FBT extract treatment increased these levels in HFD-fed mice group (Fig. 1D and E). In addition, the protein expression level of total MHC in the glycolytic muscle, extensor digitorum longus (EDL) muscle, and oxidative muscle, soleus muscle, decreased in HFD-fed mice compared to the Chow group, and FBT extract treatment increased it compared to the HFD group (Fig. S1).

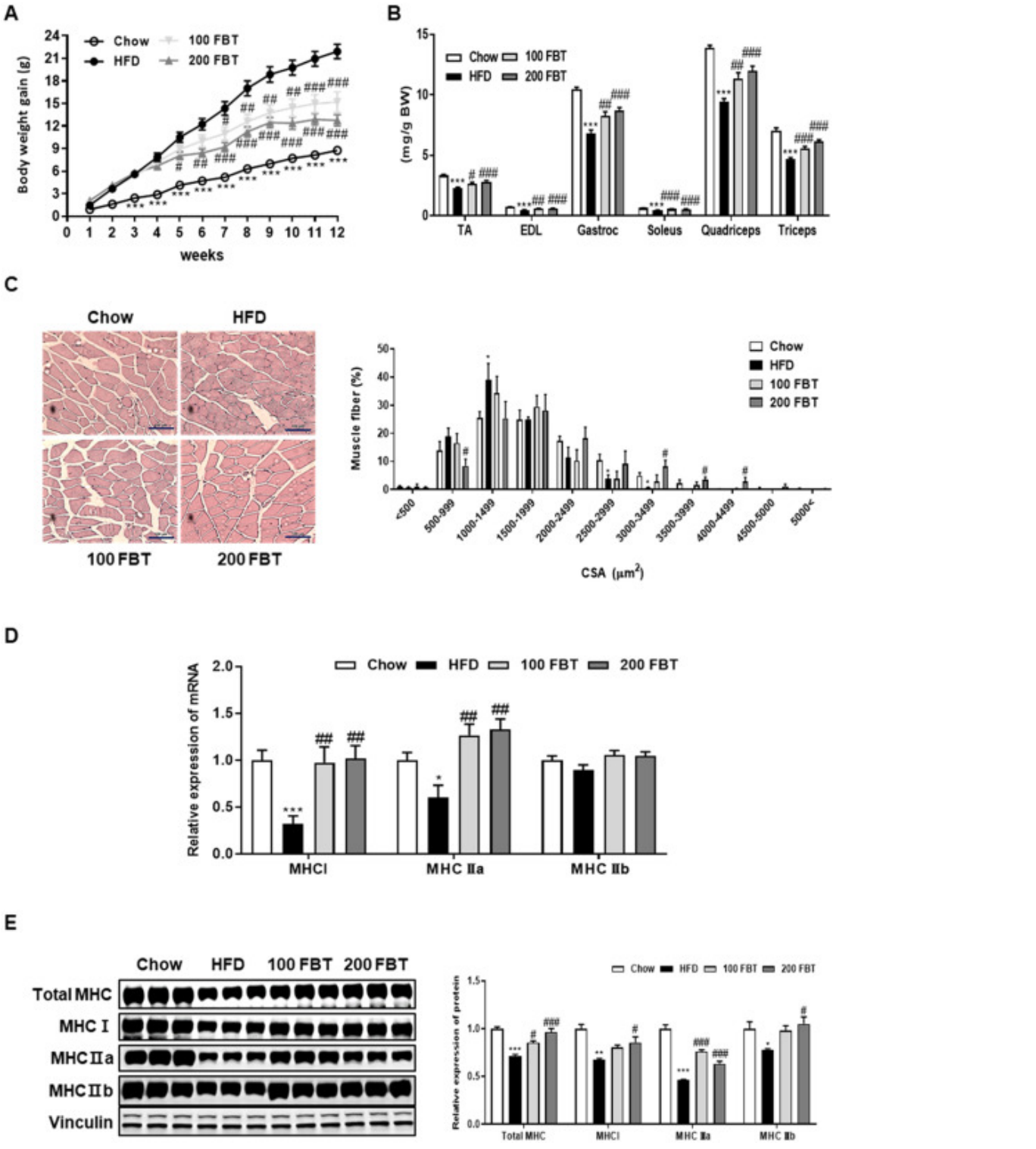

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Fig. 1. **Fuzhuan brick tea (FBT) extract increased the weight and size of skeletal muscles and myosin heavy chain (MHC) isoforms in obese mice.** (A) The body weight gain in mice from 1 to 12 weeks (n = 10 per group). (B) The weights of skeletal muscle from the experimental mice (n = 10 per group). (C) Representative image of hematoxylin and eosin staining of myofiber cross section of gastrocnemius muscle (scale bar, 100 μm), and cross-sectional area were measured (n = 3 per group).

(D and E) The expression of total MHC, I, IIa, and IIb in gastrocnemius and quadriceps muscle as determined by qRT-PCR and western blotting, respectively (n = 7 per group). Results are expressed as mean ± SEM and statistically analyzed by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the chow group) and by One-way ANOVA (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the HFD group).

3.2. FBT extract increased grip strength and running capacity of HFD-fed mice

Grip strength and treadmill running tests were performed to investigate the muscle performance of mice. Compared to the Chow group, the HFD group showed decreased grip strength normalized to body weight, running distance, and time to exhaustion. However, FBT extract treatment significantly increased grip strength, running distance, and time to exhaustion (Fig. 2). These results indicated that FBT extract improved skeletal muscle functions in mice, as measured by grip strength and treadmill running.

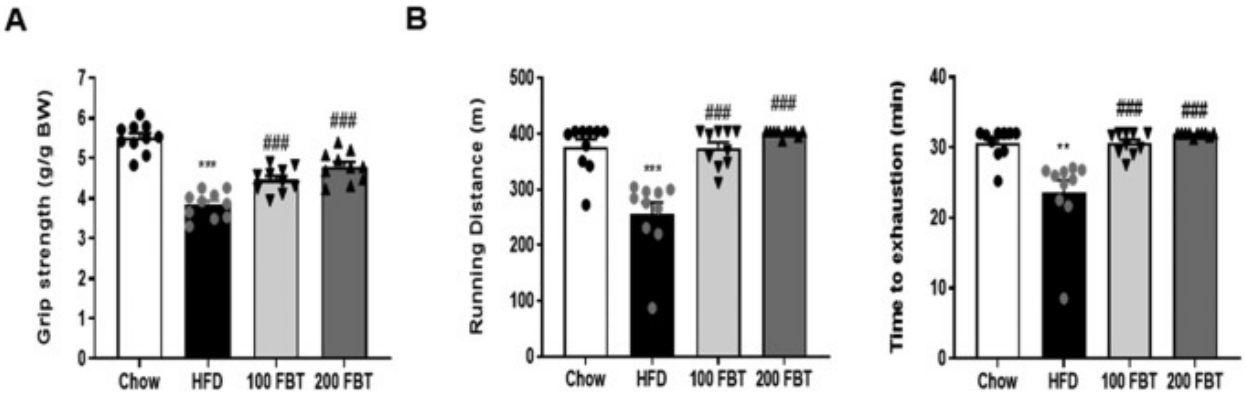

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Fig. 2. **FBT extract increased grip strength and running capacity of HFD-fed mice**. (A and B) Muscle function was measured on grip strength (g/g BW), running distance (m) and time to exhaustion (min) (n = 10 per group). Results are expressed as mean ± SEM and statistically analyzed by unpaired t-test (** $p < 0.01$, *** $p < 0.001$ compared with the chow group) and by One-way ANOVA (### $p < 0.001$ compared with the HFD group).

3.3. FBT extract decreased inflammation and atrophy-related genes expression in HFD-fed mice

Obesity increases ectopic fat deposition in muscles and causes muscle inflammation. FBT extract showed a tendency to reduce HFD-induced lipids in skeletal muscle and significantly reduced TG accumulation in skeletal muscle (Fig. 3A and B). In addition, the protein levels of JNK, p38 MAPK, c-Jun, NFκB, and STAT3 phosphorylation were increased in HFD-fed mice compared with those in chow-fed mice. However, the FBT extract group showed downregulation of phospho-JNK, p38 MAPK, c-Jun, NFκB, and STAT3 corrected for total JNK, p38 MAPK, c-Jun, NFκB, and STAT3 protein expression,

compared with the respective levels in the HFD group (Fig. 3C and D). The protein levels of TNFα and IL-6 were higher in HFD-fed mice compared with those in chow-fed mice; however, FBT extract treatment reduced the expression of these proteins in HFD-fed mice (Fig. 3D). The HFD group showed upregulated mRNA and protein levels of skeletal muscle atrophy markers, including muscle-specific E3 ubiquitin ligases, Atrogin1 and MuRF1, compared with those in the Chow group. FBT extract significantly decreased the mRNA and protein levels of Atrogin1 and MuRF1 (Fig. 3E and F). This trend was observed not only in the gastroc muscle but also in the EDL muscle and soleus muscle (Fig. S1). In addition, compared to the Chow group, the HFD group showed increased mRNA expression of myostatin, a negative regulator of muscle mass, whereas FBT extract treatment decreased its mRNA expression in HFD-fed mice (Fig. 3E).

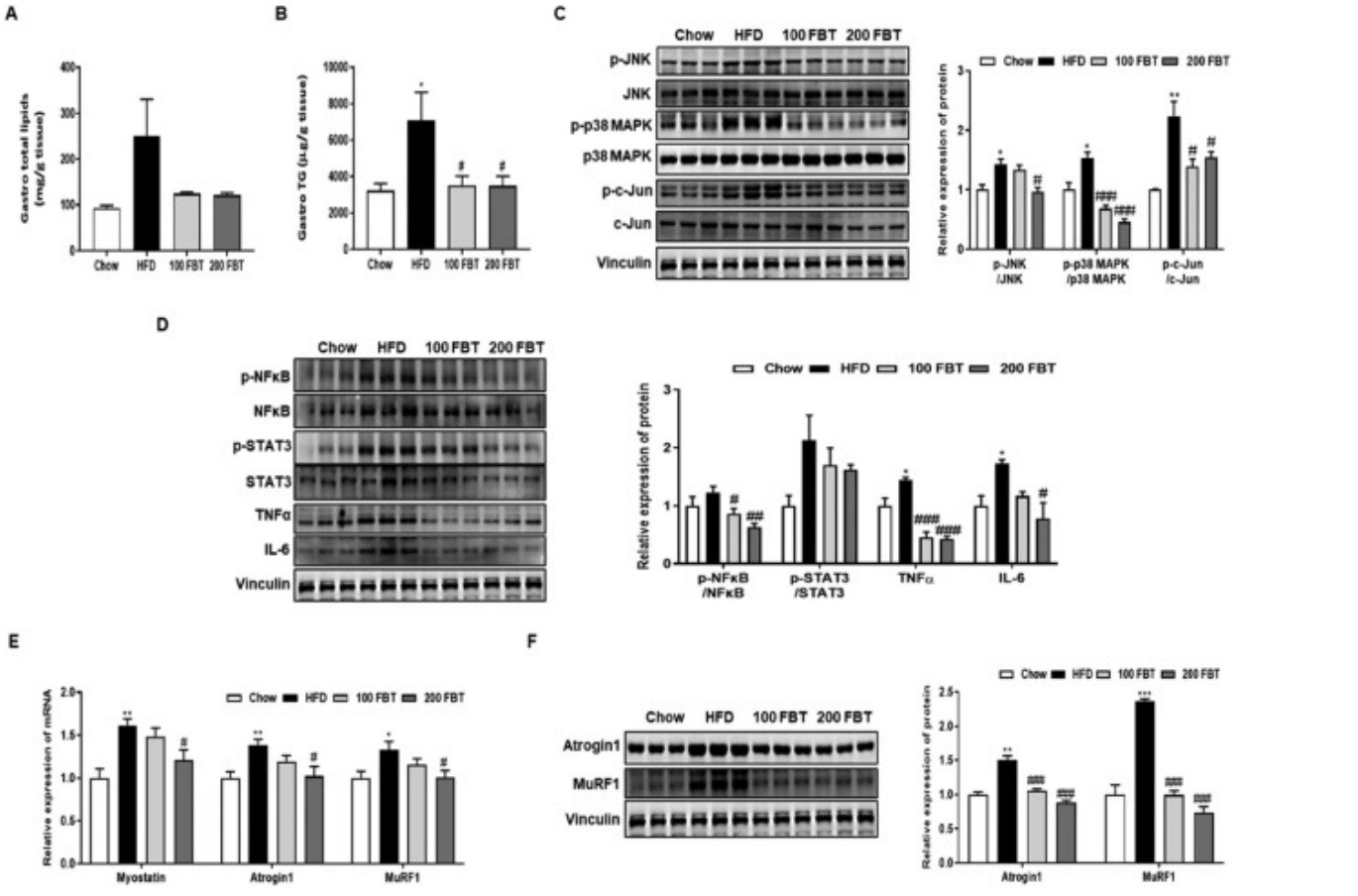

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Fig. 3. **FBT extract decreased inflammation and atrophy-related genes expression in HFD-fed mice.** (A and B) Total lipid and triglyceride levels in gastrocnemius muscle (n = 7 per group). (C) The expression of p-JNK, JNK, p-p38 MAPK, p38 MAPK, p-c-Jun, c-Jun, (D) p-NFκB, NFκB, p-STAT3, STAT3, TNFα, IL-6, and Vinculin in gastrocnemius muscle as determined by western blotting (n = 7 per group). (E and F) The expression of Myostatin, Atrogin1, and MuRF1 in gastrocnemius muscle as determined by qRT-PCR and western blotting (n = 7 per group). Results are expressed as mean ± SEM and statistically analyzed by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the chow group) and by One-way ANOVA (# $p < 0.05$, ### $p < 0.001$ compared with the HFD group).

3.4. FBT extract upregulated fatty acid oxidation-related genes and FNDC5/irisin expression in skeletal muscles of obese mice

To investigate whether FBT extract regulates fatty acid oxidation in skeletal muscles, we measured the mRNA expression levels of fatty acid oxidation-related genes in skeletal muscles of obese mice. The HFD group showed decreased mRNA expression of PPARα, PPARδ, carnitine palmitoyltransferase-1b (CPT1b), and uncoupling protein 3 (UCP3) compared with those of the Chow group. However, FBT extract increased mRNA expression of these proteins in HFD-fed mice. In addition, mRNA expression level of cluster of differentiation 36 (CD36) was higher in the HFD group than that in the Chow group, but it was decreased by FBT extract in HFD-fed mice. However, there was no change in mRNA expression of acyl-CoA synthetase long chain family member 1 (ACSL1) and CPT2 (Fig. 4A). In addition, the protein levels of AMPK phosphorylation were decreased in HFD-fed mice compared with those in chow-fed mice. However, the FBT extract group showed upregulation of phospho-AMPK corrected for total AMPK protein expression, compared with the respective levels in the HFD group (Fig. 4B). Moreover, the mRNA and protein levels of muscle FNDC5 and serum irisin were lower in HFD-fed mice than those in chow-fed mice. However, FBT extract treatment increased FNCD5 expression and tended to increase serum irisin levels of the HFD group (Fig. 4C and D).

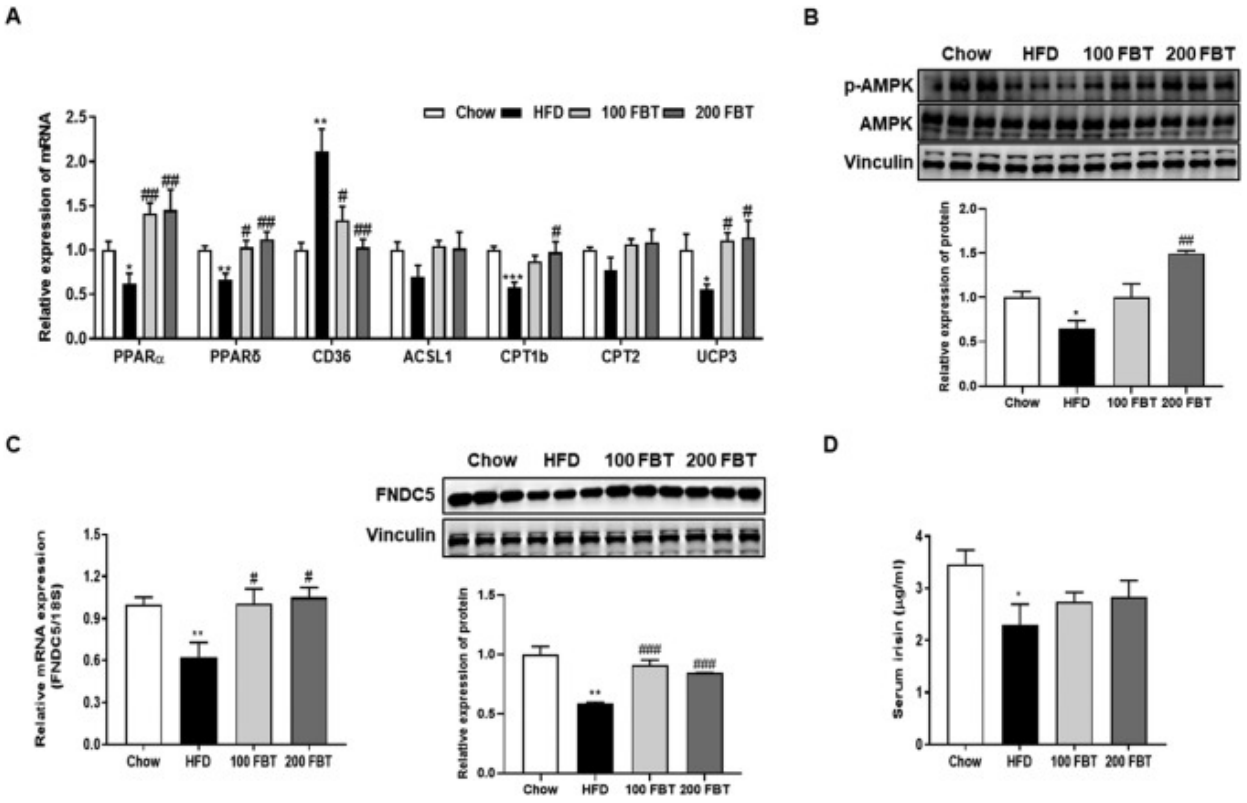

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Fig. 4. **FBT extract upregulated fatty acid oxidation-related genes and fibronectin type III domain-containing 5 (FNDC5)/irisin expression in skeletal muscles of obese mice.** (A) The expression of PPARα, PPARδ, CD36, ACSL1, CTP1b, CPT2, and UCP3 in gastrocnemius muscle as determined by qRT-PCR (n = 7 per group). (B) The expression of p-AMPK, AMPK, and Vinculin in gastrocnemius muscle as determined by western blotting (n = 7 per group). (C) The expression levels of FNDC5 in gastrocnemius muscle as determined by qRT-PCR and western blotting (n = 7 per group). (D) The serum levels of irisin (n = 7 per group). Results are expressed as mean ± SEM and statistically analyzed by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the chow group) and by One-way ANOVA (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the HFD group).

3.5. FBT extract improved mitochondrial functions, dynamics, and biogenesis in skeletal muscles of obese mice

To investigate the effect of FBT extract on mitochondrial functions, we first analyzed the mitochondrial morphology using TEM. Compared to the Chow group, HFD-fed mice showed abnormal cristae formation and swollen mitochondria. However, mitochondria size and defects such as cristae disruption and matrix density were alleviated in the FBT extract group (Fig. 5A and B). As shown in Fig. 5C-E, mitochondrial complex I and citrate synthase activities, and ATP content were significantly decreased in skeletal muscles of the HFD group compared with those in the Chow group. However, the FBT extract group showed an increase in complex I and citrate synthase activities, and

ATP content compared to those of HFD-fed mice. In addition, we measured the effect of FBT extract treatment on cellular respiration rate through OCR measurement in C2C12 cells. PA treatment significantly reduced basal and maximal respiration, ATP production, and spare respiratory capacity when compared with BSA-treated myotubes. However, FBT extract treatment significantly increased the maximal and spare respiratory capacity of PA-treated C2C12 cells (Fig. 5F). We then analyzed the expression of OXPHOS genes in skeletal muscles and observed an increase in protein levels of these genes in the FBT extract group (Fig. 5G). Western blot analysis of proteins related to mitochondrial dynamics revealed that the protein levels of Mfn1 and Mfn2, which are associated with mitochondrial fusion, were lower in the HFD group than in the Chow group, whereas FBT extract treatment led to an increase in these levels in HFD-fed mice. However, there was no change in the level of OPA1 protein. In addition, the protein levels of Drp1 and Fis1 associated with mitochondrial fission were higher in the HFD group than those in the Chow group, whereas FBT extract treatment led to a decrease of these levels in mice fed HFD (Fig. 5H). We measured the protein expression of PGC1α, which is the master regulator of mitochondrial biogenesis and respiratory function, and PGC1α-regulating genes. HFD feeding decreased the protein expression of PGC1α, ERRα, ERRγ, NRF1, NRF2, and TFAM compared to Chow-fed mice. However, FBT extract significantly increased their protein levels in skeletal muscles compared with those in the HFD group (Fig. 5I).

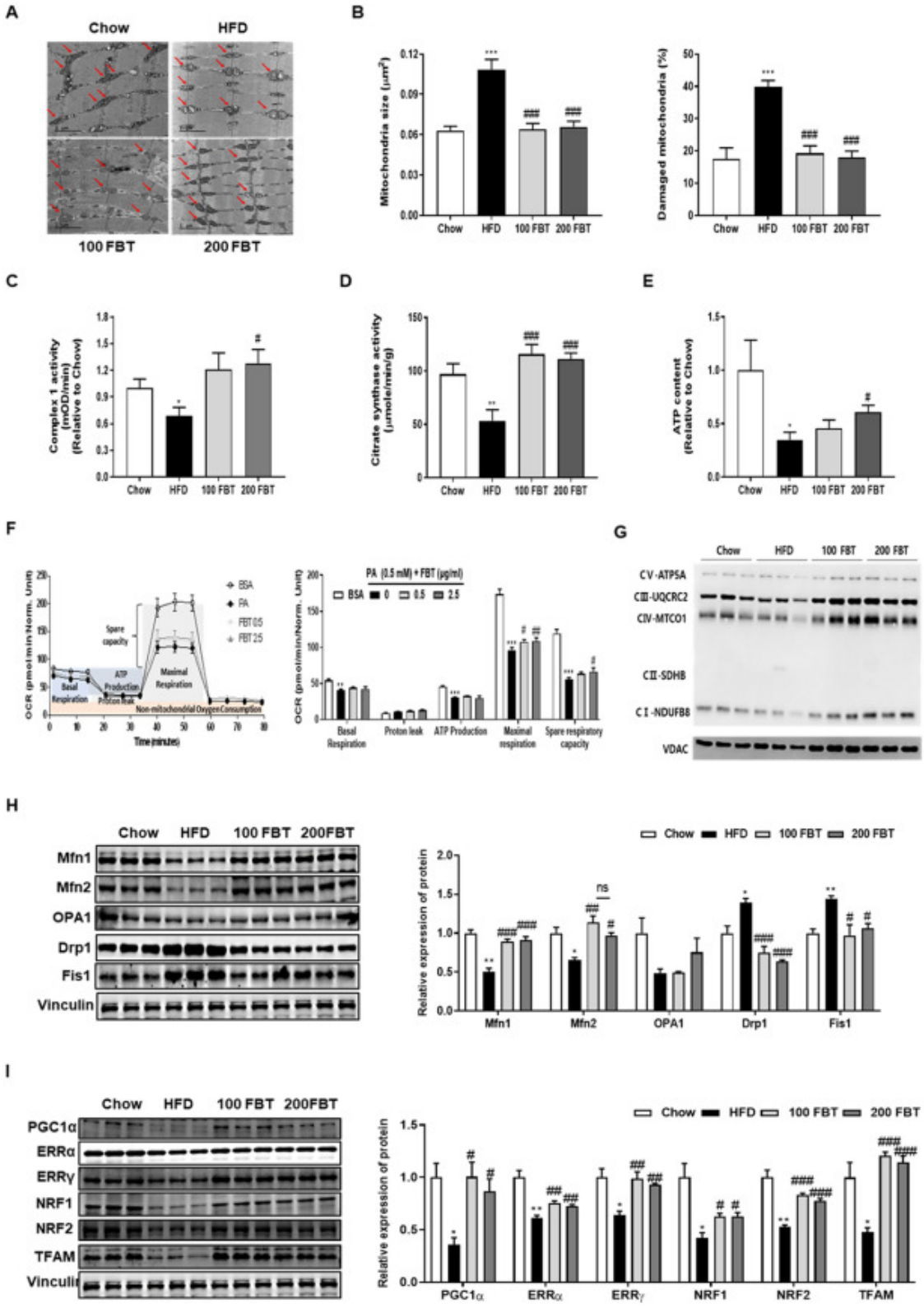

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Fig. 5. **FBT extract improved mitochondrial functions in C2C12 cells and skeletal muscle of obese mice.** (A) Representative TEM image of mitochondria morphology in gastrocnemius muscle of the mice (n = 3 per group). Arrows indicate mitochondria. (B) Mitochondria size (μm^2) and percentage of damaged mitochondria. (C) Complex I enzyme, (D) citrate synthase activities, and (E) ATP content in

gastrocnemius muscle of the mice (n = 7 per group). (F) Oxygen consumption rate (OCR) in C2C12 cells (n = 5 per group). (G) Western blotting analysis of mitochondrial OXHPOS in gastrocnemius muscle (n = 7 per group). (H) The expression of Mfn1, Mfn2, OPA1, Drp1, Fis1, (I) PGC1α, ERRα, ERRγ, NRF1, NRF2, TFAM, and Vinculin in gastrocnemius muscle as determined by western blotting (n = 7 per group). Results are expressed as mean ± SEM and statistically analyzed by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the chow group or BSA-treated myotubes) and by One-way ANOVA (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with the HFD group or PA-treated myotubes).

3.6. EGC ameliorated muscle atrophy markers, mitochondrial OCR, and proteins related to mitochondrial dynamics and biogenesis in PA-treated C2C12 cells

We investigated the impact of EGC, a key bioactive constituent of FBT extract, on obesity-induced muscle atrophy in C2C12 cells treated with PA. In comparison to BSA-treated control cells, PA treatment led to a significant reduction in the fusion index and myotube diameter, as evaluated through immunofluorescence staining for total MHC antibody. Conversely, EGC treatment resulted in a significant increase in the fusion index and myotube diameter (Fig. 6A). Moreover, PA treatment in C2C12 cells led to an upregulation of MuRF1 mRNA expression compared to BSA-treated cells, while treatment with EGC significantly reduced the mRNA expression of MuRF1 in C2C12 cells (Fig. 6B). In addition, we measured the effect of EGC treatment on cellular respiration rate through OCR measurement in C2C12 cells. EGC treatment significantly increased the PA treatment-induced decrease in the maximal and spare respiratory capacity (Fig. 6C). We observed a decrease in Mfn1 and Mfn2, and an increase in Drp1 and Fis1 protein levels in PA-treated C2C12 cells, which was reversed by EGC treatment (Fig. 6D). We observed a decrease in PGC1α, ERRα, ERRγ, NRF1, and NRF2 protein levels in PA-treated C2C12 cells, which was reversed by EGC treatment (Fig. 6E).

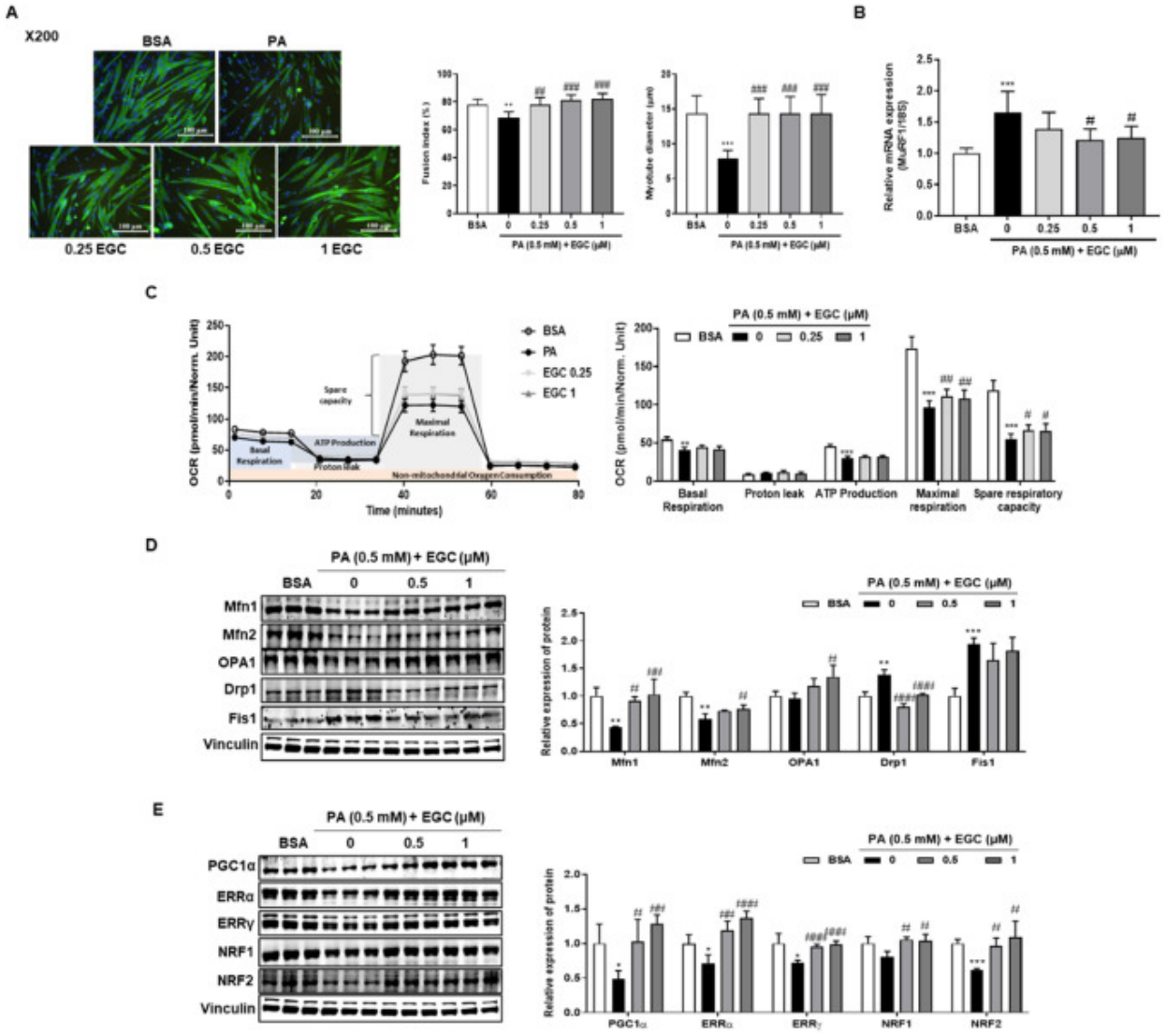

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Fig. 6. **Epigallocatechin ameliorated muscle atrophy and mitochondrial dysfunction in PA-treated C2C12 cells.** (A) C2C12 cells were fixed and stained with total MHC antibody (scale bar, 100 μm) and fusion index and myotube diameter were calculated. (B) The expression levels of MuRF1 quantified by qRT-PCR in PA-treated myotubes. (C) OCR in C2C12 cells. (D) The protein levels of Mfn1, Mfn2, OPA1, Drp1, Fis1**,** (E) PGC1α, ERRα, ERRγ, NRF1, NRF2, and Vinculin measured by western blotting in PA-treated C2C12 cells (n = 5 per group). Results are expressed as mean ± SD and statistically analyzed by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with BSA-treated myotubes) and by One-way ANOVA (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with PA-treated myotubes).

4. DISCUSSION

In this study, we examined whether FBT extract ameliorated obesity-induced muscle atrophy. Compared to the Chow group, HFD-fed mice were found to have decreased weight and size, increased lipid content and inflammatory response, and increased mRNA and protein levels of Atrogin1 and

MuRF1 in skeletal muscles. However, FBT extract treatment of HFD-fed mice increased the weight and size of skeletal muscles, while reducing TG content, inflammation, and atrophy markers in skeletal muscles of mice. These results are consistent with the finding that obesity impairs muscle protein synthesis and increases muscle protein breakdown by increasing the levels of pro-inflammatory cytokines [22]. These findings indicate that FBT extract is effective in preventing obesity-induced muscle atrophy. In addition, intramuscular lipids induce muscle lipotoxicity, leading to reduced muscle strength and power in the elderly [23,24]. In this study, FBT extract significantly increased skeletal muscle functions as measured by grip strength and treadmill running in HFD-fed mice.

Intramuscular lipids reduce mitochondrial biogenesis and cause dysfunctions such as reduced lipid β-oxidation and lipolysis [24], and the resulting toxic lipid metabolites impair muscle protein synthesis [25]. In the present study, FBT extract treatment increased the mRNA expression of PPARα,δ, CPT1b, and UCP3 and decreased CD36 in HFD-fed mice. Here, PPARα and PPARδ are well-known ligand-activated nuclear receptors that regulate the transcriptional regulation of fatty acid metabolism [26,27], and fatty acid oxidation is regulated at multiple steps from cellular uptake to mitochondrial transport to β-oxidation. When fatty acid uptake into cells is facilitated by the fatty acid transporter CD36, it is catalyzed by ACSL1, activated to COA esters, and converted to carnitine esters by CPT1b for transport into the mitochondria. Inside the mitochondria, it is converted back to acyl-CoA by CPT2 and degraded through β-oxidation, a cyclic process consisting of four enzymatic steps [28]. Thus, changes in the mRNA expression of these genes in the FBT extract group suggest an improved regulation of fatty acid metabolism.

Moreover, FBT extract treatment increased muscle FNDC5 protein expression and tended to increase the levels of secreted serum irisin. In skeletal muscles, FNDC5 protein is cleaved and secreted as a new myokine called irisin, which enhances the browning of white fat in mice by increasing the expression of UCP1 [29]. Our previous study confirmed an increase in UCP1 expression in adipose tissues of FBT extract-administered mice and reported that FBT extract prevented obesity by enhancing energy expenditure [17]. FNDC5/irisin is also metabolically involved in skeletal muscle itself, and irisin-dependent increase in AMPK phosphorylation and fatty acid uptake have been reported in cultured human muscle cells [30,31]. In present study, FBT extract treatment increased AMPK phosphorylation in HFD-fed mice, suggesting that FNDC5/irisin may play a metabolic role within skeletal muscle. Therefore, future studies are warranted to investigate the impact of FBT extract on the interaction between skeletal muscle and adipose tissue through FNDC5/irisin.

Our previous study has shown that increased energy expenditure is characterized by an increased mitochondrial content, which contributes to adipocyte thermoregulation. Moreover, HFD-fed mice exhibited elevated levels of glucose and insulin in serum [17]. This suggests that obesity-induced changes in skeletal muscle, primarily responsible for insulin-stimulated glucose disposal [32], may lead to reduced glucose uptake and increased lipid infiltration, ultimately resulting in insulin resistance. It is well-documented that insulin resistance can have a detrimental effect on mitochondrial function [33]. Nevertheless, we hypothesized that the FBT extract would have a

positive effect on mitochondrial function, given its ability to lower elevated glucose and insulin levels in HFD-fed mice. As a result, we investigated the effects of FBT extract on obesity-induced muscle atrophy, particularly focusing on obesity-induced mitochondrial dysfunction. This dysfunction encompasses decreased mitochondrial protein content, decreased enzymatic activity of the tricarboxylic acid cycle or electron transport chain, decreased mitochondrial biosynthesis, and increased mitochondrial loss [34]. In this study, we observed that FBT extract treatment increased complex I and citrate synthase activities, ATP content, and expression of PGC1α and OXPHOS gene-related proteins. Here, PGC1α has been demonstrated to be a master regulator of mitochondrial biogenesis and OXPHOS gene expression through co-activating transcription factors such as ERRα,γ and NRF1,2 [35,36]. Taken together, FBT extract appears to alleviate mitochondrial dysfunction in skeletal muscles of obese mice.

In addition to these factors, obesity impairs mitochondrial dynamics and alters the balance between mitochondrial fusion and fission, leading to mitochondrial dysfunction, such as decreased OXPHOS and ATP content in skeletal muscles [37]. In mitochondrial fusion, the outer membrane proteins Mfn1 and Mfn2 and the inner membrane protein OPA1, which are dynamin-related GTPases, play essential roles. Mitochondrial fission is primarily regulated by Drp1, which is a large GTPase. Drp1 is a cytoplasmic protein that can be recruited to the outer mitochondrial membrane to divide the mitochondria and interact with mitochondrial receptor proteins such as Fis1 [38]. In the present study, FBT extract increased Mfn1 and Mfn2 mRNA expression and decreased Drp1 and Fis1 mRNA expression. These results are consistent with those of several previous studies. Liu et al. [39] reported that Mfn1 and Mfn2 protein levels were decreased in skeletal muscles following HFD consumption, whereas Fis1 and Drp1 protein levels were increased. Jheng et al. [40] reported that the levels of mitochondrial fission protein (Drp1 and Fis1) were significantly increased in mice with HFD-induced obesity. In particular, Mfn2 is a key target of the nuclear coactivator PGC1α [41]. This suggests that the regulation of mitochondrial dynamics by FBT extract in obese mice is partially affected by PGC1α, which is upregulated by FBT extract.

Tea contains many bioactive chemicals but is particularly rich in catechins [42]. Previously, as a result of non-targeted analysis using ultra-high performance liquid chromatography-quadrupole time-of-flight-mass spectrometry to identify compounds in FBT extract, eight major compounds were identified, including gallocatechin (GC), EGC, epigallocatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), rutin, nicotiflorin, and astragalin. Among these, EGC was the most prominent [17]. This seems to be due to the fact that gallate catechins such as ECG and EGCG are gradually decomposed during the fermentation process of FBT extract, and the remaining major compound, EGC, is increased [43]. Therefore, as EGC appeared to be the most prominent compound in FBT, we conducted an experiment to explore the possibility that EGC was the major bioactive component of FBT. In this study, EGC attenuated PA-induced muscle atrophy, mitochondrial OCR, and expression of proteins related to mitochondrial dynamics and biogenesis in C2C12 cells. In addition, Kim et al. [44] reported that EGC increased the transcription of adiponectin and UCP1 in mature adipocytes. Taken together, these findings demonstrate the potential of FBT extract, particularly EGC, as a bioactive compound for alleviating skeletal muscle atrophy and mitochondrial dysfunction.

Meanwhile, among the eight major compounds mentioned, EC, EGCG, and rutin have already been reported to be effective in alleviating muscle atrophy [45], [46], [47]. Furthermore, EGC and ECG have been reported to increase skeletal muscle cell differentiation [48]. Therefore, there are five potential compounds, including EGC, that are expected to have effects on obesity-induced muscle atrophy, and additional research on other compounds appears to be necessary. Furthermore, it is necessary to further study the synergistic effect of EGC and other bioactive compounds of FBT extract on skeletal muscle atrophy.

In conclusion, our results suggest that FBT extract attenuates obesity-induced muscle atrophy and improves mitochondrial functions in skeletal muscles of mice. We identified EGC as a major bioactive compound in FBT extract and demonstrated its ability to improve PA-induced muscle atrophy and mitochondrial functions in C2C12 cells. These results suggest that FBT extract has beneficial effects on the prevention and treatment of obesity-induced muscle atrophy.

Author Contributions

A.Y. wrote original draft manuscript and performed the experimental work as well as data analysis. T.Y.H. wrote the manuscript and was responsible for study conception and design. J.A, H.D.S, JH.H, C.H.J, and S.Y.L contributed to discussion and analyzed the data. All authors reviewed and approved the manuscript.

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Author Statement

The authors declare that the work described has not been published previously (except in the form of an abstract and academic thesis), that it is not under consideration for publication elsewhere, and that its publication is approved by all authors.

Declaration of Competing Interest

The authors have no competing interest to declare.

Appendix. Supplementary materials

Download : Download Word document (206KB)

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